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ERK5 Activates NF-κB in Leukemic T Cells and Is Essential for Their Growth In Vivo

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MAPK cascades play a central role in the cellular response to the environment. The pathway involving the MAPK ERK5 mediates growth factor- and stress-induced intracellular signaling that controls proliferation or survival depending upon the cell context. In this study, we show that reducing ERK5 levels with a specific small hairpin RNA 5 (shERK5) reduced cell viability, sensitized cells to death receptor-induced apoptosis, and blocked the palliative effects of phorbol ester in anti-Fas Ab-treated cells. shERK5 decreased nuclear accumulation of the NF-κB p65 subunit, and conversely, ectopic activation of ERK5 led to constitutive nuclear localization of p65 and increased its ability to trans activate specific reporter genes. Finally, the T lymphoma cell line EL-4, upon expression of shERK5, proliferated in vitro, but failed to induce s.c. tumors in mice. Our results suggest that ERK5 is essential for survival of leukemic T cells in vivo, and thus represents a promising target for therapeutic intervention in this type of malignancy. The Journal of Immunology, 2006, 177: 7607–7617.

M itogen-activated protein kinase pathways play a central role in the signaling network that turns extracellular stimuli into distinct intracellular responses. Each MAPK cascade contains a core of sequentially activated kinases that are evolutionarily conserved in eukaryotes. Four major MAPK pathways have been identified in mammalian cells. The three canonical MAPK pathways, namely the ERK1/2, JNK, and p38 cascades, mediate proliferative and stress signaling in normal and leukemic T cells. In contrast, the role of the ERK5 cascade, which is present in normal and primary T cells (1), is unknown. The core of this pathway involves the kinases MEK kinase kinase 2 or MEK kinase kinase 3-activating MEK5, which in turn specifically activates ERK5 (2). ERK5 shares the TEY activation motif with other ERKs, whereas its other structural features are unique, such as the large regulatory C terminus that controls its nucleocytoplasmic shuttling (3). Diverse extracellular stimuli, including epidermal growth factor, serum, oxidative stress, Ag-receptor engagement, cytokines, and hyperosmolarity, induce activation of the MEK5-ERK5 module (1, 4, 5).

ERK5 mediates proliferative signaling by ErbB (6, 7), Ras (8), serum (9), insulin-like growth factor (IGF)3II (10), Bcr-Abl (11), and IL-6 (12). Accordingly, different groups have described a role for the ERK5 cascade in transformed cells: it plays a significant role in proliferation of breast cancer cells (6, 9); its overexpression is linked to bony metastases and a poor prognosis in human prostate cancer (13); it is necessary for chemoresistance in breast cancer cells (14); and it mediates cell survival in human lung cancer cells (15). More germane to our studies, ERK5 is expressed in myeloma cells, where its inhibition blocks proliferation and facilitates apoptosis induced by dexamethasone (12). Finally, ERK5 expression is essential for survival of leukemic cells expressing Bcr/Abl (11).

In vivo, invalidation of the ERK5 or MEK5 genes affects both embryonic angiogenesis and cardiac development and leads to embryonic lethality by day 11.5 (16–18). The important function of ERK5 in endothelial cells is confirmed by targeted deletion of ERK5 in adult mice, which perturbs vascular integrity and leads to death through endothelial failure (19). In cardiac tissue, activation of ERK5 inhibits injury after myocardial ischemia and reperfusion (20). ERK5 is also required for tumor-associated neoangiogenesis. In ERK5flox/flox mice carrying the Mx1-Cre transgene, deletion of the host ERK5 gene reduces the volume of tumor xenografts and leads to a significant decrease in vascular density (21). These data suggest that ERK5 plays a central role in several processes linked to tumorigenesis: transduction of intracellular oncogenic signals, tumor-associated angiogenesis, and in some cases invasion/metastasis.

Few direct targets of the ERK5 cascade have been identified. Like other MAPKs, specific docking domains mediate interactions between ERK5 and its partners (22). ERK5 activates the myocyte enhancer factor (MEF)2 family of transcription factors, leading to c-Jun expression. Interestingly, ERK5 is not redundant with the ERK1/2 cascade, because the two pathways cooperate to induce a NF-κB reporter gene in NIH3T3 cells, whereas ERK5 itself had a

3 Abbreviations used in this paper: IGF, insulin-like growth factor; CHX, cycloheximide; DN, dominant negative; FSC, forward scatter; SSC, side scatter; HA, hemagglutinin; IKK, IκB kinase; MEF, myocyte enhancer factor; PKC, protein kinase C; RSK1, ribosomal S6 kinase 1; shERK5, small hairpin RNA ERK5; wt, wild type.
minor effect (23). NF-κB transcription factors appeared as important players in the balance between cell death and cell survival, and constitutive NF-κB activity has been observed in many cancer cells (24).

In this study, we show that the oncogenic role of ERK5 in leukemic T cells depends on its ability to activate NF-κB through retention of p65 in the nucleus, allowing leukemic T cells to resist apoptotic stimuli in vitro and in vivo.

Materials and Methods

Abs and reagents

The anti-human CD3, anti-mouse CD3, and anti-mouse CD28 mAbs were purified, as described previously (25, 26). The anti-CD28, CD69, and anti-protein kinase C (PKC) mAbs were from BD Biosciences. The rabbit polyclonal Abs against ERK5 were from Cell Signaling Technology or have been described (6). The p65 and p50 rabbit polyclonal Abs were from Santa Cruz Biotechnology. The monoclonal anti-hemagglutinin (HA) (3F10) was from Roche. Donkey anti-rabbit or sheep anti-mouse IgG Abs were obtained from Amersham, and the goat anti-hamster IgG was from Pierce Biotechnology. Annexin V-PE was from BD Pharmingen. The anti-Fas Ab was from Upstate Biotechnology.

Plasmids

The expression vectors for ERK5 and ERK5 mutant (T218/Y220F) resistant to MEK5 activation (ERK5 AEF), constitutively active MEK1 (MEK1SS3) and MEK5 (S313D/T317D, termed MEK5DD), the IκB kinase (IKK) superrepressor (27), along with the c-jun and the TRE reporter (9), have been described. The expression vectors for ERK2, β-galactosidase, AP-1 and NF-AT reporters, and IL-2 promoter have been previously used (28, 29). The conventional NF-κB reporter is based on a NF-κB binding site in the IκBα chain promoter (30). pRc-hRel consists of pRcCMV with c-Rel and cDNA inserted in the HindIII-Xhol site (31). The 3× κB thymidine kinase luc reporter plasmid contains a trimer of the NF-κB-binding motif of the H-2Kb gene upstream of the thymidine kinase minimal promoter and the herpes virus thymidine kinase promoter (32). The pcDNA3p65 and pcDNA3p50 expression plasmids were provided by J. Alcamí (Hospital 12 de Octubre, Madrid, Spain). The GAL4-p65 and 5×Gal4 Luc plasmids were provided by L. Schmitz (Zentrum Ludwig-Maximilians-Universität, Munich, Germany) (33). Primers containing 19 bases of ERK5 coding sequence (AGCTGCCCTGCTCAAGTCT) or a corresponding scrambled sequence were ligated into pRetroSUPER vector to generate the small hairpin RNA ERK5 (shERK5) expression plasmid (11). The same primers were subcloned into pSIREN-RetroQ-puro (BD Biosciences) and used to create the EL-4 stable cell lines.

Cell culture and transfection

The cell lines EL-4, Jurkat T, and Jurkat T stably expressing IκBαDN (dominant negative) (34) were grown in RPMI 1640 (Invitrogen Life Technologies), 50 mM Tris-HCl (pH 8), 5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol, and incubated on ice for 10 min. The lysate was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was preincubated by incubation 30 min at 4°C with protein G agarose beads and incubated 90 min at room temperature with 1 μg of anti-HA (3F10) Ab. Twenty microliters of a 50% slurry of protein G agarose beads was added to the reaction and incubated for additional 30 min. Beads were washed three times with immunoprecipitation buffer and resuspended in 30 μl of SDS-PAGE sample buffer, denatured, separated by SDS-PAGE, and immunoblotted, as described below.

Reporter assays

In all experiments, cells were transfected with a β-galactosidase reporter plasmid (28). Transfected cells (1 × 10⁶) were harvested after 2 days and washed twice with PBS. Cells were lysed in 100 μl of luciferase lysis buffer (Promega), and luciferase assays (40 μl) were performed, according to the manufacturer’s instructions (Promega), using a Berthold luminometer (9). For β-galactosidase assays, 40 μl of lysates was added to 200 μl of β-galactosidase assay buffer (50 mM phosphate buffer (pH 7.4), 200 μg of O-nitrophenyl-β-galactopyranoside, 1 mM MgCl₂, and 50 mM 2-ME), and the absorbance was measured at 400 nm. The results are expressed as luciferase units normalized to the corresponding β-galactosidase activity. The expression level of the transfected proteins was routinely control by immunoblot analysis.

IL-2 measurement

Cells were cultured at 1 × 10⁶ cells/ml in RPMI 1640 and stimulated as indicated with anti-CD3 Abs or PMA plus ionomycin. Supernatants were harvested, and IL-2 was measured using detection kit (BD Pharmingen). The cytokines’ values were expressed as picograms per milliliter.

Immunoblotting

Cells were washed with PBS and lysed in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE on minigels and processed for immunoblot analysis, as described previously (28).

Cell cycle

Cells were washed and resuspended in 400 μl of propidium iodide staining solution (50 μg/ml propidium iodide, 0.1% trisodium citrate dihydrate, 0.1 mg/ml RNase A, and 0.1% Triton X-100). After an overnight incubation, the cell cycle profile was determined with a FACSCalibur flow cytometer (BD Biosciences).

Subcellular fractionation

For preparation of nuclear extracts, cells were washed twice in cold PBS, resuspended in 400 μl of 10 mM HEPES (pH 7.4), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, and protease inhibitors (Roche), and incubated on ice for 10 min. Nonidet P-40 was added to the cells to a final concentration of 1% (v/v). After 30 min on ice, the cell suspension was mixed by agitation for 20 min at 4°C, the insoluble fraction was removed by centrifugation at 13,000 × g for 10 min, and the soluble proteins were analyzed by immunoblotting.

Generation of stable cell lines

A total of 5 × 10⁴ EL-4 cells was cultured at 1.5 × 10⁶ cells/ml. One milliliter of supernatant from 293T cells expressing the retroviral vector pSIREN for shERK5 or control plasmid was added 1 h after plating. The same protocol was repeated 1 day later. Three days later, cells were cultured with 2.5 μg/ml puromycin (Sigma-Aldrich). After 1 wk of selection, surviving cells were isolated and kept on selection medium until used.

Cell survival and apoptosis assays

Jurkat T cells were transfected with pSUPER-GFP or pSUPER-GFP-shERK5. Two days later, GFP⁺ cells were sorted and left in culture to recover for 1 additional day. These cells or EL-4 cells were treated with the indicted apoptotic agents. Where appropriate, PMA (100 ng/ml) was added to one-half of the cells 20 min before treatment. Cell survival was analyzed by forward/side scatter (FSC/SSC) or by annexin V staining, following the manufacturer’s instructions (BD Pharmingen), as previously described (35).
**CD69 and CD25 expression**

Jurkat Tag cells were transfected with pSUPER-GFP or pSUPER-GFP-shERK5. Two days later, GFP+ cells were sorted and left in culture to recover for 1 additional day. CD69 expression was measured essentially as previously described (29). Briefly, cells were transfected with the indicated vectors and harvested 2 days later. GFP+ cells were purified by FACS, washed in RPMI 1640, and suspended in 500 μl of RPMI 1640 plus 20 μl of PE-conjugated anti-human CD69 mAb for 15 min, as suggested by the manufacturer, washed twice on RPMI 1640, and analyzed by flow cytometry (FACSscan; BD Biosciences) after gating for GFP-positive cells.

**Tumor progression in vivo**

EL-4 cells were washed in PBS and resuspended to 1.5 × 10⁶ cells/ml. A total of 2.5 × 10⁵ cells was injected s.c. in the flank of C57Bl/6 mice. Tumor development was analyzed every 2 days. Tumors were measured, and the volume was calculated following the formula: V = length × (weight)/2. All the experiments involving animals were performed according to the guidelines and regulations of the Centre Nationale de la Recherche Scientifique. J. Garaude, S. Cherni, R. Hipskind, and M. Villalba have the official degree for animal experimentation delivered by the Ministère de la Recherche (France).

**Statistical analysis**

The statistical analysis of the difference between means of paired samples was performed using the paired t test. The results are given as the confidence interval (p).

**Results**

**Inhibition of ERK5 expression induces apoptosis in Jurkat cells**

The ERK5 cascade has been linked to several different types of cancer. To examine its role in leukemogenesis, we reduced ERK5 levels in leukemic Jurkat T cells by electroporation of a bicistronic vector that expresses shERK5 (11) and GFP. As a control, we used a scrambled version of this sequence. Two days after transfection, >50% of the living cells expressed GFP (Fig. 1A, first lane). These cells were purified by FACS and placed in culture for 1 day. At this point, >90% of the cells were GFP+ (Fig. 1A, second lane), and the expression of ERK5 was largely reduced, unlike that of ERK2 (Fig. 1B). At later times, the percentage of GFP+ Jurkat cells transfected with shERK5 was substantially lower than in control shRNA-transfected cells (13.6 vs 42.7% at 4 days, and 7.2 vs 23.7% at 6 days for Jurkat-shERK5 or Jurkat-control-shRNA cells, respectively). These data suggested that expression of shERK5 inhibited cell proliferation, induced cell death, or both. In the next set of experiments, we used cells 1 day after cell sorting, i.e., 3 days after transfection.

We tested the effect of shERK5 on various parameters of cell viability. First, we examined cell morphology by flow cytometric analysis of cell size (FSC) and granularity (SSC). Dying, or apoptotic, cells are smaller and more granular than viable cells. Dot plots from one experiment are shown in Fig. 1C, and the average of three independent experiments is presented in the graphic. In the absence of stimulation, shERK5-transfected cells showed a lower rate of survival (76%) compared with control-transfected cells (89%). In early stages of apoptosis, different changes occur at the cell membrane. One involves the translocation of phosphatidylserine to the outer side of the plasma membrane, which can be detected with Annexin V. In agreement with the results of Fig. 1C, shERK5-transfected cells showed increased staining by Annexin V and therefore a higher percentage of apoptotic cells (17%) than control-transfected cells (5%). This suggests that ERK5 played a role in the survival of leukemic T cells and protected them from spontaneous apoptosis.

A classical mechanism to induce apoptosis in leukemic T cell is the engagement of death receptors, of which Fas is well characterized (36). shERK5-transfected cells were more sensitive to anti-Fas treatment, developing characteristic features of apoptosis like higher granularity and Annexin V staining (Fig. 1, C and D). Therefore, blocking ERK5 expression facilitated Fas-induced apoptosis.

Treatment with the phorbol ester PMA blocks Fas-induced apoptosis in different cell types. In T cells, activation of PKCδ by PMA inhibits anti-Fas-induced cell death (35). Interestingly, shERK5-treated cells were less effectively protected from this type of apoptosis by PMA than control cells (Fig. 1, C and D), suggesting that the intracellular pathways involved in protecting leukemic T cells were also impaired in shERK5-treated cells. In fact, PMA alone induced apoptosis in a small, but consistent number of shERK5-transfected cells (Fig. 1E).

**ERK5 knockdown renders leukemic Jurkat T cells more sensitive to TNF-α**

The transcription factor NF-κB plays an antipapoptotic role in different cell lines and mediates, in part, the protective effect of phorbol esters on Fas-induced apoptosis (34, 37). Interestingly, PMA becomes proapoptotic if NF-κB activation is blocked in Jurkat T cells (34), suggesting that blocking ERK5 activity would have a similar proapoptotic effect in leukemic cells as blocking NF-κB activation. TNF-α only induces apoptosis in Jurkat cells that fail to activate NF-κB (38). Moreover, p65−/− mice die from the increased sensitivity of hepatocytes to TNF-α, an effect that is reversed by inactivation of the type I TNF receptor (39). This protective effect of NF-κB requires gene expression, because compounds that block transcription or translation sensitize cells to TNF-α-induced apoptosis. Therefore, we investigated whether shERK5 sensitized Jurkat T cells to TNF-α. Because shERK5 induced apoptosis per se, we calculated the specific apoptosis induced by the different treatments by subtracting the percentage of apoptotic cells under resting conditions from that observed after treatment. In Jurkat cells transfected with the control scrambled shRNA, TNF-α or the protein synthesis inhibitor CHX induced apoptosis in 10% of the cells (Fig. 2A), whereas the two together induced >60% apoptosis. In contrast, TNF-α alone induced apoptosis in >50% of the shERK5-transfected cells. These data suggested that ERK5 was implicated in NF-κB activation.

Interestingly, CHX alone induced a similar rate of specific apoptosis in control and shERK5 cells. Because CHX blocks protein expression, we suspected that ERK5-induced NF-κB activation did not play a major role in CHX-induced apoptosis. Therefore, we tested two anticancer drugs whose proapoptotic effects were largely independent of NF-κB activation: etoposide, an antineoplastic agent that targets the DNA-unwinding enzyme topoisomerase II (40, 41), and taxol, which targets microtubules and causes mitotic arrest leading to apoptosis (42). Consistent with the idea that shERK5 targets NF-κB activation, shERK5 did not sensitize Jurkat cells to apoptosis induced by these two compounds (Fig. 2B). In contrast, it sensitizes Jurkat cells to Fas engagement, which cooperates with NF-κB inhibition to induce apoptosis (Figs. 1 and 2B) (43–45). Importantly, this effect was also observed in the mouse leukemic EL-4 T cell line (see Fig. 8).

**ERK5 knockdown does not affect cell cycle progression or activation of leukemic T cells**

The role of ERK5 in tumorigenesis could also be linked to cell proliferation. shERK5 expression in leukemic cells did not alter the cell cycle (Fig. 3A). Activation of T cells with PMA/ ionomycin induced cell cycle arrest at G0/G1, which was unaffected by ERK5 knockdown. Activation-induced IL-2 production was not inhibited in shERK5-expressing cells (Fig. 3B); similarly, these cells showed no changes in other activation markers like IL-2Rα (CD25) or CD69 (Fig. 3C). These results suggested that
ERK5 did not play a central role in T cell activation and excluded that defects in IL-2 production or in the up-regulation of its receptor could underlie the decrease in survival of shERK5-transfected cells. Therefore, ERK5 plays a significant role in leukemic T cell survival, but not in leukemic T cell activation or proliferation, suggesting that shERK5 did not block all T cell functions. Nevertheless, we cannot formally exclude that a low residual level of ERK5 is sufficient to mediate T cell activation and proliferation, but not T cell survival.

The ERK5 pathway activates NF-κB, but not AP-1 or NF-AT, in leukemic T cells

To test the possibility that the ERK5 pathway activates NF-κB in Jurkat cells, we used an ERK5 expression vector together with one
encoding a constitutively active mutant of the upstream kinase MEK5 (MEK5DD) that heavily phosphorylates and activates ERK5 in Jurkat cells (Fig. 4A) (1). Neither vector alone activated a NF-κB reporter gene in Jurkat cells. However, the combination of MEK5DD and ERK5 induced NF-κB trans activation in resting, PMA-, or PMA/ionomycin-stimulated cells (Fig. 4B). Similar results were obtained with the mouse leukemic T cell line EL-4, although the induction was lower (data not shown).

Next, we examined whether ERK5 activated other transcription factors in Jurkat T cells. In fact, we have shown previously that selective activation of the ERK5 pathway targets the c-jun promoter in leukemic T cells (1). AP-1 is composed of heterodimers of the Fos and Jun families of transcription factors. We cotransfected ERK5 and/or MEK5DD and investigated the effect of these plasmids on a PMA response element (TRE)-luciferase reporter gene (46) that is driven by four canonical AP-1 binding sites (Fig. 4C). The expression of either kinase alone showed no effect in resting or stimulated cells. Cotransfection of both kinases induced a 2-fold increase in resting cells, but had no significant effect in stimulated cells. Similar observations were obtained in Jurkat cells using a reporter driven by the collagenase promoter (47) that contains a single AP-1 response element (data not shown). ERK5 also failed to activate a NF-AT/AP-1 reporter derived from the IL-2 promoter (data not shown). These results show that ERK5 plays at best a minor role in AP-1 activation in T cells, probably through induction of c-Jun expression, suggesting that the antiapoptotic effect of ERK5 involves NF-κB activation.

**ERK5 activates NF-κB by a noncanonical pathway**

NF-κB is retained in the cytoplasm by the IκB family of proteins. Upon TCR stimulation, IκB proteins are phosphorylated by one of a number of IKKs, which leads to their ubiquitinylation and degradation. This frees NF-κB to translocate to the nucleus and activate its target genes. An IκB superrepressor (IκB-DN) that cannot be phosphorylated, and therefore is not degraded, retains NF-κB proteins in the cytosol and completely blocks their induction via IKK (27). NF-κB induction by PMA requires IKK activation, a process that was effectively blocked by IκB-DN (Fig. 5A). Interestingly, IκB-DN reduced ERK5 + MEK5DD-induced NF-κB activation by only 30% in resting cells (Fig. 5A), whereas the mutant completely abolished cooperation between these kinases and either PMA or PMA plus ionomycin. We obtained similar results in Jurkat cells that constitutively express IκB-DN (data not shown) (34). In T cells, PMA or anti-CD3 Abs activate PKCθ and thereby NF-κB. Conversely, inhibition of PKCθ function by the DN PKCθ-K409R mutant blocks PMA- or anti-CD3-induced NF-κB activation (30), but not that induced by ERK5 (data not shown). Therefore, ERK5 mainly activated NF-κB through a nonconventional pathway that cooperated with the canonical one to superinduce this transcription factor somewhere downstream of IκB.

Next, we tested whether ERK5 cooperated with other signals that induce the canonical pathway. In T cells, PMA and CD3 engagement induce a similar cascade of intracellular events, but TNF-α induces a different intracellular signaling cascade before activating the IKK complex (48). The ERK5 pathway not only cooperated with PMA, but also anti-CD3 Abs and TNF-α (Fig. 5B). This effect required MEK5-induced ERK5 phosphorylation, because a mutant of ERK5 that cannot be activated by MEK5 (ERK5 AEF; Fig. 5B insert and Ref. 9) had no effect in resting cells and failed to cooperate with any treatment (Fig. 5B). Therefore, the ERK5 cascade targets a pathway, activated by either TNF and/or the TCR, which lies downstream of IKKs.

**FIGURE 2.** ERK5 expression is essential for NF-κB-induced survival. A, shERK5 sensitizes Jurkat cells to TNF-α. A half million GFP + Jurkat cells were transfected as in Fig. 1, sorted at day 2, and treated the next day with TNF-α (100 ng/ml), CHX (2 μg/ml), or a combination of both. Twenty-four hours later, cells were stained with annexin V-PE and analyzed by flow cytometry for increase in fluorescence intensity (FL-2 height). Mean percentages ± SEM of specific apoptosis from three independent experiments are presented in the right-side graphic. Student’s t test: *p < 0.01; compare with control cells. The specific apoptosis was calculated by subtracting the percentage of apoptosis under resting conditions from the percentage of apoptosis after treatment. These values were 6 and 17% for control or shERK5 cells, respectively. B, Inhibition of ERK5 expression does not cooperate with taxol or etoposide. A total of 5 × 10^6 GFP + Jurkat cells was treated for 24 h with anti-Fas (5 ng/ml), etoposide (0.5 μM), or taxol (0.3 μM), and analyzed as in A.
The ERK5 pathway predominantly targets p65

Members of the NF-κB family bind as homo- or heterodimers to NF-κB regulatory elements, with p50/p65 representing the prototypical NF-κB complex and c-Rel playing a central role in T cell activation by TCR/CD28 costimulation. Although p65 and c-Rel contain transcriptional activation domains, p50 homodimers act as repressors (49). In Jurkat T cells, only overexpression of p65 activated our NF-κB reporter and cooperated with PMA above control levels (Fig. 6A). Moreover, cotransfected ERK5 + MEK5DD cooperated with p65, but not with p50 or c-Rel, to activate NF-κB in resting or PMA-stimulated Jurkat cells (Fig. 6A). c-Rel was active in our system, as it strongly induced a reporter controlled by a CD28 response element (data not shown).

We next investigated the effect of the ERK5 cascade on the 3×κB thymidine kinase reporter that is predominantly targeted by p65 (Fig. 6B). Cotransfection of ERK5 + MEK5DD significantly activated this reporter in resting or PMA-stimulated cells, and synergized with p65 to activate this reporter in resting or PMA-stimulated cells (Fig. 6B).

ERK5 controls p65 localization in the nucleus

The p65 protein shuttles in and out of the nucleus, where it exerts its biological activity (50). Proteins that induce p65 nuclear translocation or block its export to the cytosol will increase its transcriptional activity. To investigate whether ERK5 controlled p65 localization, we first blocked ERK5 expression with shERK5 (Fig. 7A) and stimulated the cells to induce p65 nuclear translocation (Fig. 7B). In the control scrambled shRNA-transfected cells, most of p65 localized in the nucleus after 30 min of stimulation, and half of the protein was still in the nucleus 2 h postinduction. In Jurkat cells with decreased ERK5 levels, the nuclear fraction of p65 was reduced after 30 min of stimulation, and was equal to resting cells after 120 min (Fig. 7B). JunB and ERK2 showed the expected localization, indicating that the different fractions were largely pure.

We next selectively activated the ERK5 pathway by transfection of MEK5DD and ERK5 and investigated p65 localization. As a positive control, we used TNF-α (Fig. 7C). Constitutive activation of the ERK5 pathway led to nuclear localization of p65 before stimulation without affecting that of p50 (Fig. 7C). As above, the pattern of JunB and ERK2 localization confirmed the purity of the different fractions (data not shown).

ERK5 induces the trans activation activity of p65

To investigate whether the intranuclear localization of p65 correlated with an increase in its transcriptional activity, we used an expression vector (Gal4 DBD-p65) that contains the DNA binding domain of the yeast GAL4 transcription factor fused to the trans activation domain of p65. The activity of this fusion protein is not blocked by IκB association or activated by its degradation. This construct, or the Gal4 DBD alone, was transfected into Jurkat T cells along with a 5×Gal4 Luc reporter gene, in which luciferase expression is controlled by five binding sites for Gal4. Cotransfection of MEK5DD + ERK5 increased trans activation by Gal4 DBD-p65 by >4-fold (Fig. 7D), and was more efficient than PMA or PMA/ ionomycin. Moreover, MEK5DD + ERK5 increased the trans-activating activity of p65 induced by either treatment. Taken
apoptosis in EL-4-control and EL-4-shERK5 cells (data not shown). Interestingly, ERK5 down-regulation in EL-4 stable cell lines, but not in transient transfected Jurkat cells, led to an increase in the ERK2 protein. We confirmed these results by preparing a second stable shERK5-expressing EL-4 cell line (shERK5 B) (Fig. 8A, right panel). Whereas ERK2 overexpression in these cells might compensate for the decrease of ERK5 in certain cellular functions, it does not account for the increase in apoptosis or the decrease in p65 accumulation in the nucleus (Fig. 8, B and C).

The immune system clears tumor cells by inducing apoptosis through different mechanisms. One involves activation of death receptors on the surface of tumor cells. In contrast, some tumor cells escape immune system surveillance by becoming resistant to apoptosis. Because shERK5 rendered leukemic T cells more sensitive to death receptor-induced apoptosis, we injected EL-4-shERK5, as well as control cells, in C57B6 mice and investigated tumor formation. Wild-type (wt) EL-4 cells induced tumors in all mice (Fig. 9). The tumors were first observed 1 wk after cell injection and progressed rapidly, as shown by the plots in Fig. 9. In contrast, EL-4-shERK5 cells failed to develop tumors (Fig. 9) in four independent experiments (n = 20). The second EL-4-shERK5 cell line (EL-4-shERK5 B) also failed to induce tumors (data not shown). In contrast, EL-4 cells stable expressing small hairpin RNA for luciferase

FIGURE 4. The ERK5 module targets NF-κB, but not AP-1 or NF-AT, in Jurkat cells. B and C, 10⁶ Jurkat-TAg cells were cotransfected with 3 μg of each ERK5 and/or MEK5-DD, 2 μg of the NF-κB or TRE luciferase reporters, and 1 μg of β-galactosidase expression vector. Control cells represent cells transfected with equal amounts of an empty vector. Two days later, equal numbers of cells were left unstimulated or stimulated for 4 h with PMA (100 ng/ml) or PMA plus ionomycin (1 μg/ml), and luciferase and β-galactosidase activities were evaluated. A, 10⁷ cells were analyzed by immunoblotting after anti-HA immunoprecipitation. The data represent means ± SD, n = 8, Student’s t test: **, p < 0.0005; compare with control cells.

together, our results suggest a role for ERK5 in potentiating trans activation by p65.

ERK5 is essential for tumor progression of leukemic T cells

The mouse leukemic T cell line EL-4 has been extensively used to generate tumors in C57B6 recipient mice (51, 52). We infected EL-4 cells with a viral vector expressing shERK5 and the purumycin resistance gene to obtain a stable cell line. Although the control shRNA viral vector rapidly generated puromycin-resistant cells, the shERK5 vector gave rise to very few puromycin-resistant cells. We finally obtained a cell line, called EL-4-shERK, that showed decreased ERK5 expression (Fig. 8A), increased spontaneous apoptosis relative to control cells (Fig. 8C), and grew in vitro at a comparable rate to control cells. shERK5-infected cells showed impaired PMA-induced p65 accumulation in the nucleus (Fig. 8B) and were more sensitive to Fas ligand-induced apoptosis (Fig. 8C). All of these results were in agreement with our results in Jurkat T cells, and confirmed our hypothesis that ERK5 protects leukemic T cells from death receptor-induced apoptosis. Similarly to Jurkat cells (Fig. 2), taxol, CHX, and etoposide induced comparable levels of apoptosis in EL-4-control and EL-4-shERK5 cells (data not shown). Interestingly, ERK5 down-regulation in EL-4 stable cell lines, but not in transient transfected Jurkat cells, led to an increase in the ERK2 protein. We confirmed these results by preparing a second stable shERK5-expressing EL-4 cell line (shERK5 B) (Fig. 8A, right panel). Whereas ERK2 overexpression in these cells might compensate for the decrease of ERK5 in certain cellular functions, it does not account for the increase in apoptosis or the decrease in p65 accumulation in the nucleus (Fig. 8, B and C).
but more likely reflected clearance of tumor cells. Furthermore, these results indicate that ERK2 up-regulation in shERK5-expressing cells could not compensate for the down-regulation of ERK5 in vivo.

Discussion

The ERK5 and NF-κB pathways play a significant role in the development and survival of different kinds of tumor cells. Moreover, treatments aimed at decreasing the activity of NF-κB, such as the proteasome inhibitor bortezomib, have demonstrated clinical benefit. In this study, we show that both ERK5 and NF-κB signaling cooperate to facilitate leukemic T cell survival in vitro. Furthermore, ERK5 knockdown blocked tumor progression in mice, suggesting that ERK5, in addition to NF-κB, may be an ideal candidate for therapeutic intervention, at least in T cell leukemias.

Disregulation of ERK5 has a significant role in maintenance of leukemic and perhaps other malignant cells, but our results do not show that ERK5 is necessarily oncogenic per se. Previous reports (12) in tumor plasma cells indicated that the ERK5 cascade plays a role in the control of neoplastic B cells. However, the mechanism underlying its activity was not detailed in that study. In this study, using a related cellular model derived from a T cell malignancy, we confirmed that the ERK5 pathway participates in the control of survival of leukemic T cells. Moreover, studies on the mechanism by which ERK5 may influence leukemic cell survival indicated a predominant role of the NF-κB pathway as a mediator of the action of ERK5. Several findings support this conclusion. First, blocking ERK5 expression in leukemic T cells by shRNA induced spontaneous apoptosis; second, it sensitized them to TNF-α-induced apoptosis, a process that absolutely requires inhibition of NF-κB activity (38, 39); third, it failed to sensitize Jurkat T cells to apoptotic agents that do not require NF-κB inhibition, i.e., etoposide, taxol, and CHX; fourth, it converted the survival agent PMA into an apoptotic factor, as does blocking NF-κB activity (34). Importantly, these

induced tumors like the wt cells. shERK5-injected mice sacrificed at day 17 contained no tumor cells. Moreover, mice infected with EL-4-shERK5 were healthy 2 mo after injection, showing that the absence of tumors was not related to slower proliferation,

FIGURE 6. The ERK5 module targets p65 in Jurkat T cells. A and B, 10⁷ Jurkat-TAg cells were cotransfected with 3 μg of ERK5 and MEK5DD expression vectors and/or 1 μg of the expression vectors for p65, p50, and c-Rel, together with 2 μg of the corresponding luciferase reporters and 1 μg of the β-galactosidase expression vector. Two days later, equal numbers of cells were either left unstimulated or stimulated for 4 h with PMA (100 ng/ml), and luciferase and β-galactosidase activities were evaluated. The data represent means ± SD, n = 4, Student’s t test: **, p < 0.01; compare with p65-transfected cells.

FIGURE 7. ERK5 controlled p65 nuclear localization. A and B, 6 × 10⁷ Jurkat cells were transfected with shERK5 or with a scramble sequence (control). Three days later, GFP⁺ cells were sorted by FACS (Fig. 1), and stimulated for different times with PMA (100 ng/ml) plus ionomycin (1 μg/ml). After subcellular fractionation, the presence of the different proteins in the nuclear and cytosolic fractions was analyzed by immunoblotting. C, 10⁸ million Jurkat-TAg cells were cotransfected with 2 μg of GFP and an empty vector or ERK5 plus MEK5DD expression vectors. Two days later, GFP⁺ cells were sorted and equal numbers of cells were left unstimulated or, as a positive control, stimulated for 10 min with 20 ng/ml TNF-α. Cells were fractionated, and only the nuclear fractions were analyzed for enrichment of p65 or p50 by Western blotting. D, 10⁷ Jurkat-TAg cells were cotransfected with 1 μg of ERK5 and MEK5DD expression vectors and/or 0.5 μg of the expression vectors for Gal-DBD or Gal-p65, together with 1.5 μg of the Gal4-luciferase reporter plasmid and 1 μg of the β-galactosidase control vector. Two days later, equal numbers of cells were either left unstimulated or stimulated for 4 h with PMA (100 ng/ml) and PMA plus ionomycin (1 μg/ml), and luciferase and β-galactosidase activities were evaluated. The data represent means ± SD, n = 4, Student’s t test: *, p < 0.02; **, p < 0.01; ***, p < 0.005; compare with Gal4-p65-transfected cells.
effects were also observed in the mouse leukemic cell line EL-4. In the latter, and during the generation of the EL-4 stable cell lines, we confirmed that ERK5 was essential for EL-4 survival, because few cells carried the puromycin-resistant gene, and therefore, shERK5. We probably selected cells that expressed low levels of ERK5, but still enough to survive. Knockdown of ERK5 cooperated with Fas engagement to induce apoptosis, as occurs when NF-κB activity is inhibited (43–45). It is interesting that inhibition of NF-κB also enhances TRAIL-induced apoptosis (53). TRAIL, a member of the TNF family of receptors, induces apoptosis in many transformed cells, but not in normal cells. Our preliminary results show that shERK5 renders leukemia cells more sensitive to TRAIL. In line with these findings, Aza-Blanc et al. (54) have described that blocking ERK5 expression by siRNAs enhanced TRAIL-induced death. As TRAIL is being explored as a potential anticancer drug, the development of strategies to decrease ERK5 function may be of therapeutic value by increasing TRAIL effectiveness.

Our results indicate that NF-κB activation by ERK5 occurred by a nonconventional mechanism that does not totally require IKK-mediated phosphorylation of IκB. NF-κB is composed of homo- and heterodimers of Rel family proteins. All Rel proteins share a Rel homology domain controlling DNA-binding activity. In addition, p65/RelA, RelB, and c-Rel contain a C-terminal trans activation domain. A DN mutant of IκB blocks conventional activation of NF-κB, but not that induced by ERK5, suggesting that the latter does not involve phosphorylation of IκB, but rather another, perhaps later, event. This may involve nuclear accumulation of p65. In fact, reduction of ERK5 provoked a decrease in the nuclear retention of p65, indicating that ERK5 may favor NF-κB activation. We are investigating this possibility.

Although this mechanism appears attractive, other possibilities may also explain the effect of ERK5 on NF-κB activation. We have shown previously that TCR stimulation induces accumulation of phosphorylated ERK5 in the nucleus (1). Therefore, ERK5 could control p65 localization by direct interaction. Related to this is the interaction of the C-terminal region of ERK5 with MEF2 transcription factors. This domain was required for coactivation of MEF2D by ERK5, and the MEF2-ERK5 interaction was found to be dependent upon activation. The transcriptional activation domain of ERK5 is required for maximal MEF2 activity in response to calcium flux in T cells, and it can activate the endogenous Nur77 gene when constitutively recruited to the Nur77 promoter via MEF2 sites (59). Therefore, p65 could recruit ERK5, and its transcriptional activity, to p65-dependent promoters, or vice versa.

FIGURE 8. ERK5 down-regulation in EL-4 cells inhibits p65 accumulation and facilitates death receptor-induced apoptosis. A, 10^6 control or shERK5-expressing cells were subjected to immunoblotting to analyze the expression of ERK5, ERK2, and actin. Jurkat cells were transiently transfected, and EL-4 cells were stable transfected. Right panel, Shows the results of two EL-4 stable cell lines expressing shERK5. B, 5 × 10^5 control or shERK5 cells were stimulated with PMA (100 ng/ml) plus ionomycin (1 μg/ml) for 30 min. After subcellular fractionation, the presence of the different proteins in the nuclear and cytosolic fractions was analyzed by immunoblotting. C, 2 × 10^5 wt or shERK5 were treated with Fas ligand (1 ng/ml). Twenty-four hours later, cells were stained with annexin V-PE and analyzed by flow cytometry. Mean percentages ± SEM of specific apoptosis from three independent experiments are presented. Student’s t test, *, p < 0.01; compare with control cells. Specific apoptosis was calculated by subtracting the percentage of apoptosis under resting conditions from the percentage of apoptosis after treatment. These values were 11.4 and 14.8% for control or shERK5 cells, respectively. The experiments were performed three times with similar results.

FIGURE 9. Down-regulation of ERK5 blocks tumor development in mice. A, Five C57B6 mice were injected with 2.5 × 10^5 control or shERK5 cells. Every 2 days, tumor size was measured. The wt mice with large tumors were euthanized 17 days after inoculation. Two months after cell injection, shERK5-injected mice were still alive and healthy. This experiment was performed four times with identical results (n = 20). B, Tumors of euthanized mice injected with different EL-4 cell lines: wt, expressing small hairpin RNA for luciferase or expressing shERK5 were weight and the average ± SD is depicted in the graphic.
More generally, the idea that p65 might interact with ERK5 raises the interesting possibility that ERK5 acts as a nuclear docking site for multiple transcription factors, extending their temporal nuclear activity, as occurs with MEF2.

Both NF-κB and ERK5 have been linked to the proliferation/survival of other tumor cells, opening the question as to whether these pathways also collaborate in supporting tumor growth in other cell systems. Although we have not tested the role of these pathways also collaborate in supporting tumor growth in vivo observations indicate that the ERK5-NF-κB pathway is essential for tumor-associated angiogenesis. Thus, the vascular failure induced by targeted deletion of ERK5 in adult mice arises from massive apoptosis of endothelial cells (19). Endothelial and hematopoietic cells have the same onco-genic precursor, suggesting that they probably share certain intracellular signaling mechanisms. It will be very interesting to determine whether endothelial cells derived from ERK5-deficient mice show defects in NF-κB activation by IFN-γ or other factors that promote survival in these cells.

ERK2 and ERK5 seem likely to share some biological functions. Prolonged ERK5 down-regulation could induce an increase in ERK2 levels to compensate for the reduced ERK5 levels. In stable cell lines in which ERK5 has been down-regulated by shRNA, increased ERK2 levels might enhance survival. This might explain why we do not observe these differences in transient transfections, in which there is no long-term selection underway. The data obtained in mice injected with wt or ERK5 knockdown EL-4 cells argue in favor of an in vivo role of the ERK5 pathway in the regulation of T cell leukemias. Interestingly, the in vitro proliferation properties of EL-4⋅shERK5 cells did not differ from that of wt EL-4 cells. Yet, the former were unable to create tumors in mice. This discrepancy between in vitro and in vivo behavior very likely reflects the complex physiological conditions encountered by the tumor cells in vivo. One possible explanation is increased tumor cell killing by death receptor agonists available in the animal, but absent in vitro. Whatever the mechanism, our in vivo observations indicate that the ERK5-NF-κB duos is an attractive target for therapeutic intervention in leukemias.

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Disclosures
The authors have no financial conflict of interest.

References